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Full Length Article

Chromatographic and Spectroscopic Fingerprinting of *Ficus carica* and Evaluation of *In Vitro* Antioxidant Activity

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Abstract

The present study was conducted to evaluate the *in vitro* antioxidant activity of *Ficus carica*, commonly known as fig. Methanol and ethanol extracts of *F. carica* leaves were subjected to 2, 2-diphenyl-1-picryhydrazyl (DPPH) free radical scavenging activity assay where ascorbic acid being positive control had an IC_{50} value of 3.98±0.26 while methanol and ethanol fractions showed an IC_{50} of 101.76±1.12 and 93.12±1.17 respectively exhibiting their high antioxidant potential. DPPH assay was also performed on high performance liquid chromatography (HPLC) elutions. Most active antioxidant components in ethanol extract were eluted between 17–18 min, and those in methanol were eluted over 14–15 min and upon ultra-high performance liquid chromatography-mass spectrometery (Orbitrap Liquid Chromatography-Mass Spectrometry) were identified to be 13-Docosenamide, (Z)- for ethanol and ficusin for methanol fraction. Thus, it is concluded that these two components are most probable determinants of antioxidant potential of *F. carica* leaf extracts. © 2021 Friends Science Publishers

Keywords: *Ficus carica;* Antioxidant; DPPH; HPLC; LC-MS; *In vitro*

Introduction

Ficus carica belongs to family *Moraceae*. It was originated from Western Asia and later spread to the whole world. It is a large shrub to small deciduous tree. *F. carica* is used traditionally to treat various ailments like gastrointestinal, cardiovascular and respiratory disorders (Duke 2002).

Presence of steroids, coumarins, flavones and triterpenoids in *F. carica* leaves have been reported in plants from European and Asian continents (Peyron *et al*. 2000; Saeed and Sabir 2002; Vaya and Mahmood 2006). *F. carica* leaves also exhibits anti-inflammatory, antioxidant (Ali *et al.* 2012), antimicrobial (Jeong *et al*. 2009), antidiabetic (Canal *et al*. 2000), antipyretic (Vikas *et al*. 2010) and anti-HSV activities (Wang *et al.* 2004). Oxidative stress is one of the main causes of several life threatening neurological and cardiovascular disorders worldwide (Kasote *et al.* 2015) and the progression of these health issues remains rather unchecked among South-East Asian populations. Nevertheless, the indigenous plants with antioxidant potential can serve as therapeutic agent for all populations ranging from those living in the foothills of the Himalayas to those living in metropolitan zones. Free radical accumulation is responsible for a number of pathophysiological conditions like cardiovascular and neuromuscular disorders. In current era focus is shifted to the use of natural antioxidant compounds for a number of chronic diseases because of their health protecting properties (Teixeira *et al.* 2009). Antioxidants can reduce or prevent the damage by donation of electron to the damaged cells. Synthetic antioxidants have a number of limitations. They are unstable at high temperatures, and due to their chemical structures, they are volatile, harmful in high doses. Natural antioxidants, on the other hand, are stable at high temperatures, have no impact on the color or odor of the food and have high solubility (Taghvaei and Jafari 2015). So, the search for natural antioxidants is in high demand.

Antioxidant activities of *F. carica* fruit and leaf has been reported in a few studies earlier (Ahmad *et al*. 2013a; Mawa *et al*. 2013; Moloudizargari *et al*. 2013) but the compound/s responsible for the antioxidant activity of *F. carica* were still to be ruled out. So, the specific objectives of our study were to confirm *in vitro* antioxidant potential of *F. carica* leaf extracts at first and then at next stage our goal was to further perform a comprehensive analysis of the organic extracts of *F. carica* leaves in order to authentically identify compounds responsible for antioxidant potential using latest spectroscopic and chromatographic techniques. It is already well established that a correlation exists between the amount of total phenol and flavonoid and antioxidant capacity of *F. carica* leaves (Konyalıoğlu *et al*. 2005). We hypothesized that isolated compounds responsible for strong antioxidant potential of *F. carica* leaf extract may belong either to polyphenols or flavonoids or both of them. So, we aimed to conduct a comprehensive research starting from antioxidant potential of organic

fractions of *F. carica* leaves and ending up at identifying potential compound responsible for antioxidant potential. If verified by our study it will authenticate the link between presence of polyphenols, flavonoids and antioxidant potential of *F. carica* leaves.

Materials and Methods

Plant material

After proper identification, healthy and disease-free leaves of *F. carica* were collected from Plant Genomic Research Institute (PGRI), National Agricultural Research Council (NARC), Islamabad, Pakistan, in March and April 2016. A voucher specimen (FC-AJ-001) was deposited in the herbarium of the Department of Biological Sciences, COMSATS University, Islamabad, Pakistan.

Extraction

The leaves of *F. carica* were washed gently, yet thoroughly, with deionized water and shade dried at room temperature for two weeks. Sterilized mortar and pestle and a steel grinder were used to grind the dried plants into a fine powder. Ten g of powdered leaf samples were put in 100 mL each ethanol and methanol and were sonicated for 30 min. using an ultrasonic cleaning bath (Branson Ultrasonic Cleaner 3210R-DTH, 130 V input power, frequency of 40 kHz) at 25°C. Mixtures were then filtered with filtration assembly having a pore size of 0.45 µm. The filtrate was evaporated under reduced pressure and lyophilized to obtain leaf extracts and were stored at 4°C till further use.

Free radical scavenging activity against DPPH reagent

The solution of 2,2-diphenyl-1-picryhydrazyl (DPPH) radical (0.2 m*M*) was prepared by mixing 3.94 mg of the DPPH powder to 50 mL methanol. A 96 well black plate (Corning Incorporated Costar, 3603) was used for the assay. An antioxidant assay was performed according to the protocol of Yang *et al.* (2011) with minor alterations. Crude extracts were tested for their antioxidant potential. There were three for each sample and twelve standard serial dilutions were prepared for ascorbic acid, and *F. carica i.e.* 400, 300, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125, 0.39 μg/mL. Log of concentration (μg/mL) was plotted against percent DPPH radical scavenging activity. Methanol was used as a negative control. The reaction mixture in each well of 96 well plate consisted of 100 μL sample, 100 μL DPPH (0.2 m*M*) in methanol. Experiment was conducted in the dark and absorbance was measured at 517 nm, using a Spectra Max M5 Spectrophotometer (Molecular Devices) after 30 min incubation. Percent scavenging activity (SA%) was calculated using the formula (Yang *et al.* 2011):

$$\text{SA (\%)} = \frac{A^{C}_{517\ nm} - A^{S}_{517\ nm}}{A^{C}_{517\ nm}} \times 100\%$$

Where $A^{C}_{517\ nm}$ and $A^{S}_{517\ nm}$ are the absorbance at 517 nm of the control and a sample, respectively. Graphing and data analysis were carried out with Origin Software. Log of concentration (μg/mL) was plotted against percent DPPH radical scavenging activity. The IC_{50} values of each raw extract were calculated by fitting the corresponding dose response curve.

High performance liquid chromatography (HPLC) analysis

Reverse Phase (RP)-HPLC fractionalization of methanol/ethanol extracts was performed on an Agilent 1260 Liquid Chromatography equipped with a quaternary solvent delivery system, an auto-sampler, and a DAD detector. A SHISEIDO CR column (100 mm × 2.0 mm) was used at an ambient temperature of 25°C for all analysis. The mobile phases consist of (A) aqueous formic acid (0.1% v/v) and (B) acetonitrile. An elution program was performed using a gradient with a flow rate of 0.2 mL/min: 5 to 20% B from 0 to10 min, 20 to 40% B from 10 to 25 min, 40 to 50% B from 25 to 40 min, 50 to 60% B from 40 to 45 min, 60 to 70% B from 45 to 58 min and 70 to 5% B from 58 to 60 min. UV absorption detection was set at 280 nm.

DPPH assay against HPLC fractions

For both ethanol and methanol extracts, the eluent of ten repeated HPLC separations were collected at each minute for all samples for up to 40 min, and ten fractions at each minute were mixed for lyophilization overnight. Then, each residue was re-dissolved with 100 μL methanol subjected to DPPH assay following the procedure described above.

Liquid chromatography and mass spectrometry (LC-MS)

LC-MS was performed on an Orbitrap Fusion Lumos mass spectrometer (Thermofisher Scientific) coupled to an Ultimate 3000 liquid chromatograph (Thermofisher Scientific). The key parameters for the negative, as well as, positive mode are given in Table 1. Orbitrap LC-MS Data analysis was performed on Xcalibur 4.0 software.

Statistical Analysis

Software Origin (Pro 8), Origin Lab Corporation, Northampton, MA, USA was used for all statistical analysis. Data were collected from three independent extractions for each fraction and reported as mean± standard deviation (SD).

Results

Free radical scavenging activity of DPPH was evaluated in 1958 (Blois 1958) and today it is the most widely used and reliable method to determine the ability of a compound to

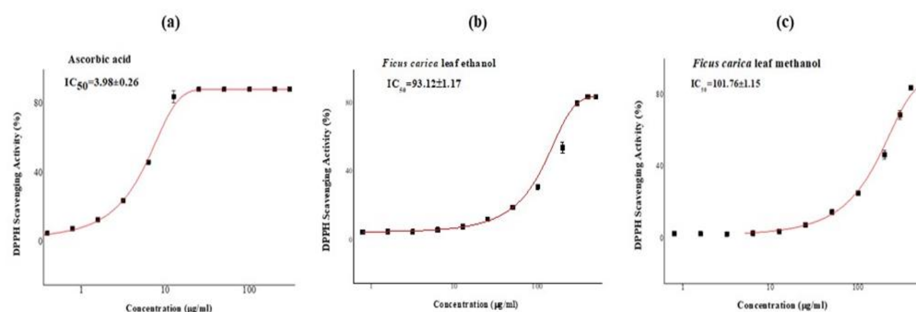

Fig. 1: Antioxidant activity of (a) ascorbic acid (b) ethanol extract and (c) methanol extract of *F. carica* leaves as characterized by DPPH assay (n= 3)

act as a free radical scavenger and hence possesses an antioxidant potential. IC_{50} values were calculated by Software Origin (Pro 8), Origin Lab Corporation, Northampton, MA, USA. A sigmoid non-linear regression fitting model was used and a curve was plotted with concentration (μg/mL) along abscissa and percent DPPH scavenging activity was plotted along ordinate. IC_{50} values of *F. carica* in methanol and ethanol were 93.12±1.17 and 101.76±1.15 μg/mL, respectively (Fig. 1b-c) as compared to ascorbic acid *i.e.* 3.98±0.26 μg/mL (Fig. 1a). The log of concentration has been plotted against % scavenging activity. IC_{50} below 50 μg/mL was considered high, IC_{50} between 50 and 100 μg/mL was considered considerably high, between 100 and 200 μg/mL was moderate and little or no activity was considered beyond 200 μg/mL.

DPPH assay against HPLC fractions

In order to identify the bioactive compounds of ethanol and methanol extracts of *F. carica* leaves, we used the DPPH assay described above to evaluate HPLC elutions. Crude methanolic and ethanolic extracts, were subjected to HPLC separately and monitored by UV-Vis detection at 280 nm (Fig. 2a-b). HPLC fractions were then collected at one min. interval up to 60 min and the antioxidant activity of each fraction was measured by using DPPH assay in the same manner as performed for crude extracts. As shown in Fig. 2c-d, the most active components in methanol fraction were eluted between 14 -15 min, and those in ethanol fraction eluted over 17 - 18 min.

Liquid chromatography and mass spectrometry (LC-MS)

Ultrahigh mass resolution mass spectrometry coupled to HPLC (LC-Orbitrap-MS) was utilized to identify the HPLC fractions with potential antioxidants. LC-MS data obtained under both positive and negative ion modes compounds identified in the HPLC fractions are shown in Table 2. According to the DPPH assay and LC-MS data, fraction of methanol eluted between 1–2 min. was identified as methoxsalen and the one eluted between 14–15 min was of ficusin. Methoxsalen was found in low abundance in methanol extract of *F. carica* leaves as measured by GC-MS (Fig. 5b). The most active component in *F. carica* ethanol fraction eluted at 17–18 min was identified to be 13-Docosenamide, (Z)- though it was detected in a low

Table 1: LC-MS parameters for positive and negative ion modes for methanol and ethanol fractions of *F. carica* leaves

Parameters	Positive mode	Negative mode
Electrospray voltage(V)	3000	2400
Sheath gas (Arb)	40	40
Auxiliary gas (Arb)	15	15
Sweep gas (Arb)	2	2
Ion transfer tube temp. (°C)	300	300
Vaporizer temp. (°C)	250	250
Detector type	Orbitrap	Orbitrap
Orbitrap resolution	120000	120000
Mass range (m/z)	150-2000	150-2000
Maximum injection times (ms)	50	50
AGC target	4e5	4e5
RF lens (%)	30	30

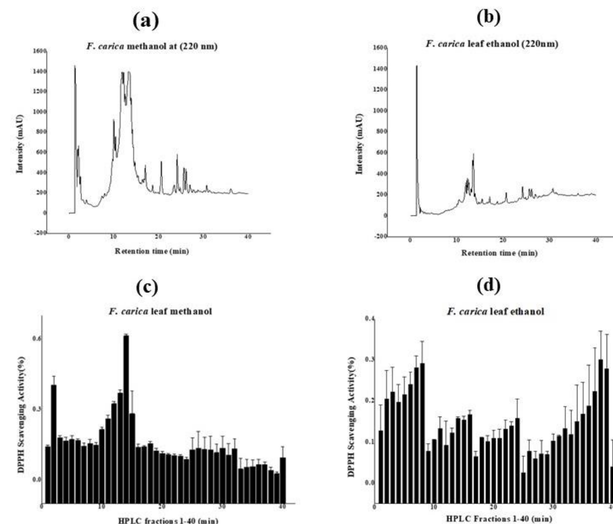

Fig. 2: HPLC Chromatogram detected at 220 nm of (a) methanol and (b) ethanol extracts of *F. carica* leaves (c, d) antioxidant activity of HPLC fractions of methanol and ethanol extract respectively as measured by DPPH assay

Table 2: Components identified in *F. carica* leaves ethanol and methanol extracts by LC-MS under positive and negative ion modes

Retention time (min)	*m/z* ($[M+H]^+/[M-H]^-$)	z	Mol. formula	Name
		F. carica methanolic extract		
16–17	217.05	1+	$C_{12}H_9O_4$	Methoxsalen
14–15	187.04	1+	$C_{11}H_7O_3$	Ficusin
30–31	279.23	1+	$C_{18}H_{31}O_2$	9,12,15-Octadecatrienoic acid (Z,Z,Z)
1–2	215.03	1-	$C_{12}H_7O_4$	Methoxsalen
		F. carica ethanolic extract		
17–18	338.34	1+	$C_{22}H_{44}O\ N$	13-Docosenamide, (Z)-
0–1	255.23	1-	$C_{16}H_{31}O_2$	n-Hexadecanoic acid
0–1	283.26	1-	$C_{18}H_{35}O_2$	Octadecanoic acid

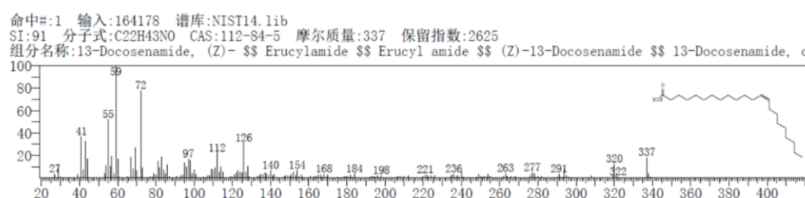

Fig. 3: HPLC Chromatogram of 13-Docosenamide, (Z), found in low abundance but with strong antioxidant potential in *F. carica* leaves

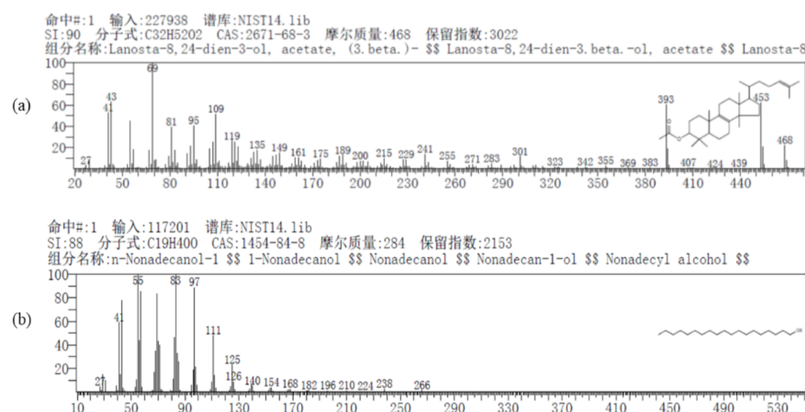

Fig. 4: Chromatograms of (a) lanosta-8,24-dien-3. beta-ol, acetate with RT 50.1min. and peak area14.93% (b) nonadecanol with RT 21.948 min and peak area 26.15%. Both compounds were found in high abundance but have low antioxidant potential in *F. carica* leaves

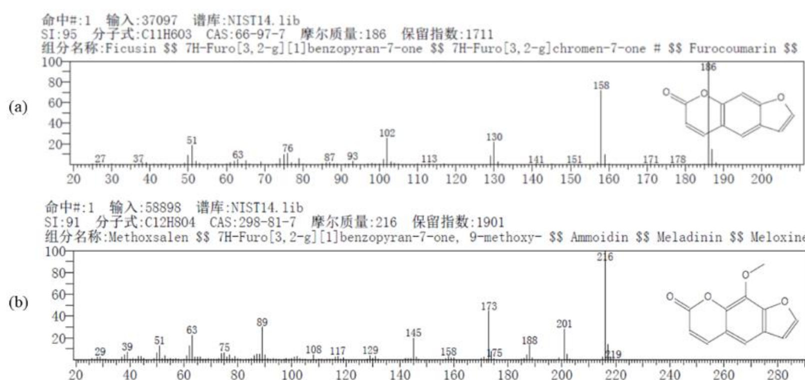

Fig. 5: Chromatogram of (a) ficusin found in low abundance - 0.53% (b) methoxsalen also in low abundance - 0.44%. Both compounds are strong antioxidant components in *F. carica* leaves

abundance in GC-MS analysis (Fig. 3). So, we concluded that although these compounds are found in lower abundance in methanol and ethanol fractions of *F. carica* leaves, they showed strong antioxidant potential as confirmed by DPPH assay of HPLC fractions and later on via Orbitrap LC-MS. The compound in ethanol fraction with highest abundance as

detected by GC-MS was lanosta-8, 24-dien-3. beta. -ol, acetate with area under the peak being 14.93% and retention time (RT)= 50.1 min (Fig. 4a). Similarly, the compound with highest abundance in methanol extract was nonadecanol with RT 21.948 min and peak area 26.15% (Fig. 4b). Ficusin is found in low abundance in ethanol fraction 0.53% (Fig. 5a). Methoxsalen was present in low abundance 0.44% in methanol fraction (Fig. 5b).

Discussion

The DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants (Oyaizu 1986). Strong antioxidant potential of *F. carica* leaf has already been reported (Konyalıoğlu *et al.* 2005; Mahmoudi *et al.* 2016). As, mentioned earlier it is known that a correlation is present between amount of total phenol and flavonoid and antioxidant capacity of *F. carica* leaves (Konyalıoğlu *et al*. 2005). But an uncertainty was present about the identification of phenol or flavonoid responsible for the antioxidant potential of *F. carica* leaves. In our findings, ficusin found in *F. carica* methanol extract were the main flavonoid responsible for antioxidant potential of *F. carica*. Methoxsalen on the other hand is a furanocoumarin which was also identified in methanol extract of *F. carica* leaf. In a Moroccan study, strong antioxidant potential of *F. carica* was reported (Ayoub *et al*. 2019), it has also been established that *F. carica* leaves are rich sources of polyphenols at all stages of development (Nadeem and Zeb 2018), possessing strong antioxidant potential. Exhibition of strongest antioxidant potential by ficusin in our study verifies this claim and also shows that our results are comparable to previous reports about *F. carica* being a strong antioxidant candidate and ficusin, being a polyphenol, validate previous studies *i.e. F. carica* leaves are rich source of polyphenol.

Methanolic extract of *F. carica* leaves also exhibited similar results, having scavenging inhibition of 4.11, 8.10 and 10.22% at a concentration of 10, 150 and 250 μg/mL respectively (Ahmad *et al.* 2013). A strong antioxidant activity of *F. carica* leaf aqueous-ethanol extract was attributed to the presence of phenolic compounds like methoxypsoralen, rutin, psoralen, dipentoside, dihydoxybenzoic acid and oxypeudacin (Belguith-Hadriche *et al.* 2017). In another research, 30% of the total antioxidant activity of *F. carica* leaf, determined via DPPH assay is due to the phenolic compounds present (Teixeira *et al.* 2009). Strong antioxidant activity of *F. carica* methanol leaf extract was reported in another previous study (Ergül *et al.* 2019). Our current and previous reports suggest the possible role of *F. carica* leaves in prevention and reduction of free radicals. This potential thus can be translated into a protective role of *F. carica* in chronic diseases.

For herbal drug discovery spectroscopic analysis is the mainstay and hence one of the most important step. This knowledge can lead to the development of new leads which help in designing of new molecules with little modifications. HPLC plays a main role in isolation, separation and fingerprinting of herbal samples and for structure elucidation, mass spectrometry is the technique of the choice. These powerful techniques are revolutionizing the field of natural herbal drug discovery. Thus, the knowledge gained with current study can be used further to design a curative antioxidant drug which is easily accessible, cost effective and has no or fewer side effects. Future perspectives of our study are to determine anti-inflammatory potential of *F. carica* leaves and conduct animal model trails.

Conclusion

Our study is unique in the aspect that antioxidant compound has been determined in *F. carica*. Results not only validated this information but also gave an insight into the possible antioxidant compounds using Orbitrap LC-MS. The knowledge imparts progress in drug designing with the purpose to introduce the therapeutic remedies for most common ailments through indigenous resources. Identification of specific natural antioxidants from *F. carica* leaves in the present study may give new insights for establishment of biologically derived antioxidants. The folklore usage of this native plant *F. carica* by traditional healers is also justified.

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Author Contributions

AJ designed the study, conducted experiments, collected data, performed analytical methods, drafted manuscript. SN conceived the presented idea and supervised the research work. SA and ZA improved write-up. All authors discussed the results and gave critical feedback thus leading to achieve the current form of the manuscript.

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